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Aristolochic acid nephropathy: A worldwide problem

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Aristolochic acid nephropathy (AAN), a progressive renal interstitial fibrosis frequently associated with urothelial malignancies, was initially reported in a Belgian cohort of more than 100 patients after the intake of slimming pills containing a Chinese herb, *Aristolochia fangchi*. Although botanicals known or suspected to contain aristolochic acid (AA) were no longer permitted in many countries, several AAN cases were regularly observed all around the world. The incidence of AAN is probably much higher than initially thought, especially in Asia and the Balkans. In Asian countries, where traditional medicines are very popular, the complexity of the pharmacopoeia represents a high risk for AAN because of the frequent substitution of the botanical products by AA-containing herbs. In the Balkan regions, the exposure to AA found in flour obtained from wheat contaminated with seeds of *Aristolochia clematitis* could be responsible for the so-called Balkan-endemic nephropathy. Finally, despite the Food and Drug Administration's warnings concerning the safety of botanical remedies containing AA, these herbs are still sold via the Internet.

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Aristolochic acid nephropathy (AAN), a rapidly progressive interstitial nephritis leading to end-stage renal disease and urothelial malignancy, was originally reported in Belgium in a group of patients who had ingested slimming pills containing powdered root extracts of Chinese herbs (Figure 1a and b).[1–4] This nephropathy, initially called Chinese-herb nephropathy (CHN), appeared to be the dramatic consequence of a substitution of *Stephania tetrandra* by *Aristolochia fangchi* rich in aristolochic acid (AA), because both herbs share the same common name in Pin Yin (Han Fang Ji and Guang Fang Ji), and one can be used instead of the other in traditional Chinese medicine irrespective of their botanical classification.[1,3,5] After the publication of the index cases, new cases of AAN were regularly reported, not only in Belgium but also worldwide (Figure 1a).[1–4,6–23] Actually, the true incidence of AAN is largely unknown and probably underestimated, as numerous ingredients known or suspected to contain AA are used in traditional medicine in China, Japan, and India (Figure 1c).[24–26]

Another reason to suspect a higher number of AAN cases is based on the hypothesis that AA could be an environmental cause of Balkan-endemic nephropathy (BEN), which is a familial chronic tubulointerstitial disease characterized by an insidious onset, a slow progression to end-stage renal disease, and an increased frequency of urothelial cancer.[23] This nephropathy is endemic in Serbia, Bosnia, Croatia, Bulgaria, and Romania (Figure 1d).[27]

Finally, despite the Food and Drug Administration's warnings regarding the safety of botanical remedies containing AA (known or suspected to contain AA), plants containing AA are still available via the Internet.[28]

AAN: THE BELGIAN OUTBREAK OF CHN

In 1992, two young women with no previous history of renal disease were admitted in our Nephrology department in Brussels (Belgium) with severe interstitial nephritis that progressed over a couple of months to end-stage renal disease.[1] One year before, these two patients had followed the same weight-loss program in the same medical clinic in Brussels. Interestingly, the composition of weight-reducing pills was modified in June 1990 by introducing root extracts from two Chinese herbs, labeled as *S. tetrandra* and *Magnolia officinalis*.[1] An epidemiological survey of the Nephrology centers of Brussels revealed an unusual, increased incidence of patients with 'interstitial nephritis of unknown origin'

admitted for dialysis in 1991 and 1992.[1] This survey identified seven other women who had followed the same slimming regimen containing the Chinese herbs.[1] Confronted to this rapidly progressive nephropathy probably related to the ingestion of these two Chinese herbs, the Belgian authorities decided to ban *S. tetrandra* and *M. officinalis* from the Belgian market at the end of 1992. Despite this safety measure, more than 100 cases of CHN were reported in Belgium in 1998, 70% of them being in end-stage renal disease.[7]

The time correlation between the introduction of the Chinese herbs in weight-loss regimens and the appearance of this rapidly progressive interstitial renal fibrosis focused the search of the culprit on the Chinese herbs.[1] Very soon, the inadvertent replacement of *Stephania* by *Aristolochia* was suspected. First, *S. tetrandra* (Pin Yin name: Han Fang Ji) and *A. fangchi* (Pin Yin name: Guang Fang Ji) belong to the same therapeutic 'Fang Ji' family in traditional Chinese medicine, and the herbal ingredients are generally traded using their common Pin Yin name. Second, the pathological aspect of CHN is very similar to that of BEN, whose etiology is still controversial, but some suggested that AAs containing *A. clematitis* are the main culprit (see section below).[1,2,29,30] The hypothesis of substitution was strengthened by the phytochemical analyses of the *S. tetrandra* batches revealing that most of them did not contain tetrandrine but AAs ($0.65 \pm 0.56\ mg\ g^{-1}$ of powder), the main compounds of the *Aristolochia sp.*[3] A survey including 71 CHN/AAN patients

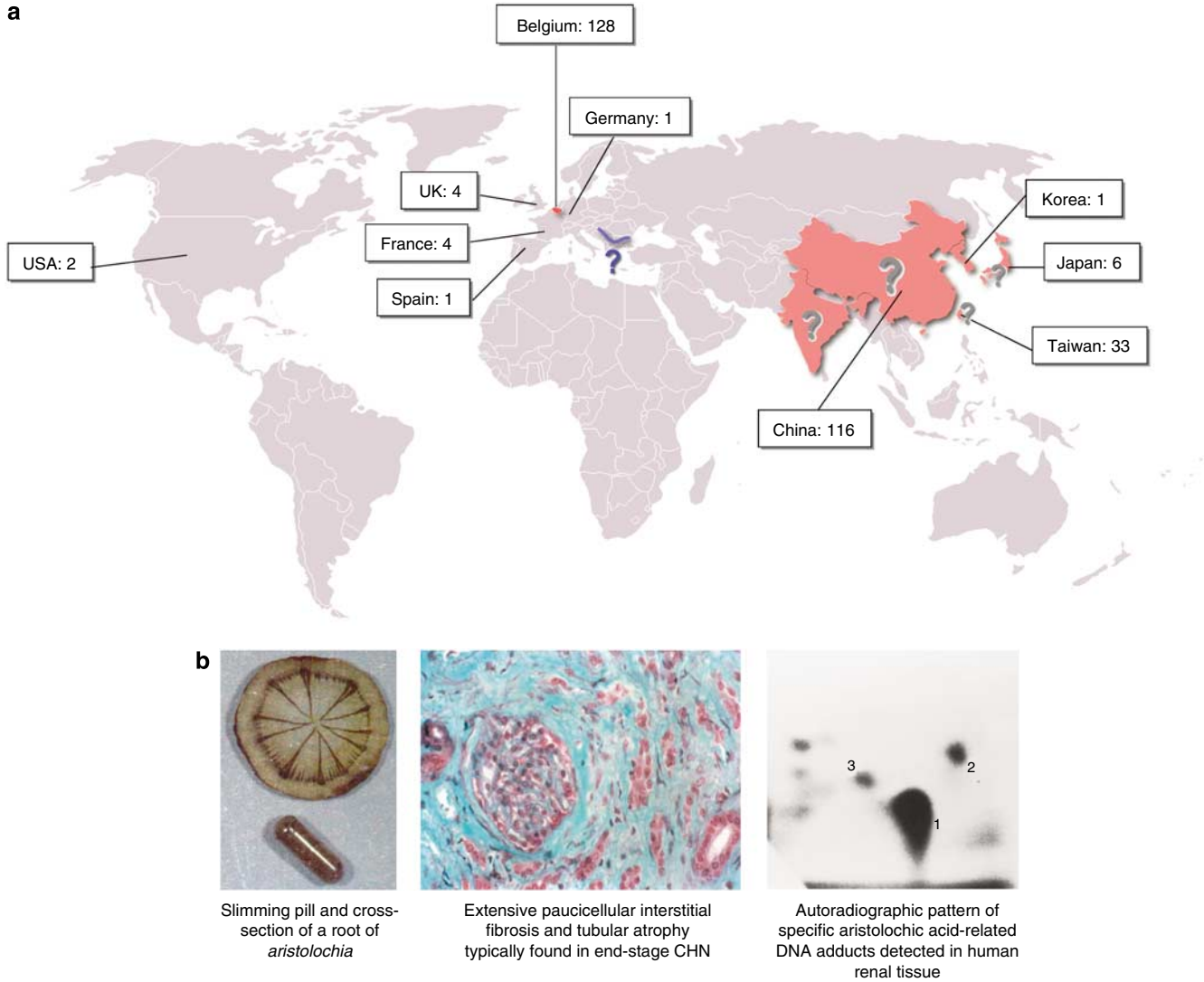

Figure 1 | Aristolochic acid nephropathy: a worldwide problem. (**a**) Counting cases of CHN/AAN around the world reported in the literature.[1–4,6–23] (**b**) CHNA/AAN is a rapidly progressive interstitial nephritis leading to end-stage renal disease and urothelial malignancy, which was originally reported in Belgium in the context of the intake of slimming pills containing powdered Chinese herbs (*A. fangchi*). (**c**) The true incidence of AAN is largely unknown and probably underestimated, as numerous ingredients known or suspected to contain AA are used in traditional medicines in India and Eastern Asia (see Tables 1–3 for more details). (**d**) Finally, another reason to suspect a higher number of AAN cases is based on the hypothesis that the exposure to seeds of *Aristolochia clematitis* comingled with wheat grain during the annual harvest could be responsible for BEN.

c

A pharmacy selling traditional herbal remedies including Fang Chi and Mu Tong

d

Aristolochic acid containing *Aristolochia clematitis* growing in a field of wheat (Croatia)

By courtesy of Dr Bojan Jelaković

Figure 1 | Continued.

followed in our department demonstrated in a multiple regression analysis that the cumulative dose of the so-called *Stephania* (in fact, *Aristolochia*) was the only significant factor predicting the slope of the inverse of plasma creatinine levels.[31] The causal role of AA was definitively confirmed by the detection of AA DNA adducts in kidney removed from CHN/AAN patients showing evidence of a previous exposure to AA.[32,33] In addition, the main histological and functional features of CHN/AAN were successfully reproduced by administrating AA to New Zealand white rabbits or male Wistar rats.[34,35] Consequently, the term Chinese-herb nephropathy has been progressively abandoned and replaced by aristolochic acid nephropathy.

After the first report of cases in Belgium, other similar cases were sporadically observed in France, Spain, Germany, United Kingdom, and the United States, in the context of herbal remedies for slimming purposes but also for all kinds of indications such as eczema, hepatitis B, 'liver enhancement', arthritis, rheumatism, and pain relief.[6,8–10,13,15,17,20] As expected, numerous cases were also reported in Asian countries where the complexities of the traditional pharmacopoeia represent a high risk for AA exposure.[12,14,16,19,21,22,36–40]

Clinically, the initial presentation of CHN/AAN was usually silent and the renal failure was discovered by routine blood testing.[41] However, few cases presenting with a Fanconi syndrome or an acute renal failure due to tubular necrosis were reported in the literature.[11,12,15,19,21] Anemia was present and was often more severe than might have been anticipated from the degree of renal failure.[42] In most of the cases, urinary sediment was unremarkable and dipstick analysis for albuminuria was negative.[43] However, urinary excretion of five low molecular weight proteins (β2-microglobulin, cystatin C, Clara cell protein, retinal-binding protein, and α1-microglobulin) was markedly increased in five patients with CHN/AAN, and the urinary low molecular weight protein/albumin ratio was higher than in control patients with glomerular diseases.[44] Moreover, in 26 patients with CHN/AAN, levels of urinary neutral endopeptidase, a 94 kDa ectoenzyme of the proximal tubule brush border, were significantly decreased in those with moderate renal failure and almost undetectable in those with end-stage renal failure.[45] NEP enzymuria positively correlated with individual creatinine clearance values ($r = 0.76$; $P = 0.0001$) and negatively correlated with urinary low molecular weight protein levels ($r = -0.55$; $P = 0.00001$).[45] An *in vitro* study on the opossum kidney cell line demonstrated that AA intoxication led to a rapid and persistent decrease in megalin expression in parallel with an inhibition of receptor-mediated endocytosis of low molecular weight protein.[46] Taking together, these data indicated that proximal tubular cells are the main target of the AA-containing Chinese herb.

Macroscopically, the kidneys were shrunk, asymmetric in about half of the cases with irregular outlines in one-third.[43] Microscopically, an extensive interstitial fibrosis with atrophy and loss of proximal tubules was the predominant lesion, which was mainly located in the superficial cortex and progressed toward the deep cortex (Figure 1b).[2,29] The glomeruli were relatively spared, although, in the later stage of the disease, they displayed a mild collapse of the capillaries and a wrinkling of the basement membrane. Although it was not the general rule, an interstitial inflammatory infiltration was retrieved in several renal biopsy specimens, suggesting an immunological process as a possible pathophysiological mechanism. This observation was the rational basis for a pilot study with corticosteroids performed in 35 CHN/AAN patients with chronic renal failure. Compared to an untreated group, a significant reduction of the number of patients reaching end-stage renal disease was observed after 1 year of steroid therapy.[47] Eight years later, in a larger group of CHN patients, the steroid therapy was confirmed to slow down the progression of renal failure.[48]

Finally, the finding of an endothelial wall thickening in the interlobular and afferent arterioles suggested a possible ischemic process induced by other substances concomitantly administrated with the Chinese herb.[49] The appetite suppressant (dex)fenfluramine, a serotonin agonist, could have played a role in the development of CHN because serotonin injection was experimentally shown to induce ischemic renal lesions progressing to renal fibrosis.[50] However, this hypothesis can be ruled out on the basis of reports of CHN cases out of the context of a slimming regimen and the demonstration that dexfenfluramine did not enhance the nephrotoxicity of AA in a rat model of CHN/AAN.[41,51] In the same line, an extrarenal toxicity of Chinese herbs was also suspected because 30–50% of the CHN patients displayed an aortic insufficiency.[43] However, the puzzling association of valvular abnormalities with CHN appeared to be mainly linked to the concomitant presence of anorectic drugs in the

slimming pills, with the demonstration of a significant dose–response relationship between the cumulative dose of (dex)fenfluramine and the aortic regurgitation.[52,53]

AA CONTAINING HERBAL REMEDIES IN TRADITIONAL MEDICINES

The uncontrolled use and the uncorrected identification of medicinal herbs, which are usually considered by the general population as inherently harmless, were at the heart of all AAN cases encountered in the Western countries.[54]

On the other hand, in Asia, AAN was the dramatic consequence of the complex pharmacopoeia of herbal remedies used in traditional medicines and the lack of their regulation as applied for conventional drugs.[5] For example, two series of 12 and 20 AAN cases, respectively, related to the use of various herbal medications were reported in Taiwan.[14,16] The ingestion of AA-containing herbal remedies used in traditional Sino-Japanese Kampo prescriptions (vernacular names: Boui and Mokutsu) resulted in Fanconi syndrome secondary to AAN in four Japanese patients.[11,12] In China, acute renal failure secondary to tubular necrosis was observed in eight patients after the intake of Guanmutong (*Aristolochia manshuriensis Kom.*), an AA-containing Chinese herb widely used for the treatment of urinary and cardiovascular diseases.[21] Two series of 58 and 51 AAN cases, respectively, were reported in 2001 in China, most of them after the intake of the liver tonic Longdan Xieganwan that contained Caulis *Aristolochia Manshuriensis*.[22] Five additional AAN cases were also observed in Hong Kong, following the substitution of the nontoxic Herba Solani Lyrati by the AA-containing herb *Aristolochia mollissimae*.[22]

The preferential use of vernacular names in traditional medicine terminology and the high risk for substitution of the herbal products might explain the outbreak of AAN in Asia (for more details, see Tables 1–3). However, one can expect that the true incidence of AAN in Asia is largely underestimated. Indeed, *Akebia* is commonly used in Sino-Japanese prescriptions as well as Fang ji and Mu Tong in traditional Chinese medicine.[24,25] It is worthy to stress that traditional medicine is still widely popular in China in 2007: about 3000 hospitals provide traditional Chinese medicine treatments to nearly 234 million patients each year.[60] Despite the fact that the AA-containing herbs were theoretically banned in many countries around the world, including

Table 1 | Botanicals known or suspected to contain aristolochic acid and their vernacular names[55–59]

Botanical name	Common or other names
Aristolochia spp.	Aristolochia, Guan Mu tong, Guang Mu tong
Aristolochia acuminata (Syn. *Aristolochia tagala*)	Oval leaf Dutchman's pipe
Aristolochia bracteata	Ukulwe
Aristolochia clematitis	Birthwort
Aristolochia contorta	Ma Dou Ling (fruit), Bei Ma Dou Ling (root), Tian Xian Teng (herb)
Aristolochia cymbifera	Mil homens
Aristolochia debilis (Syn. *Aristolochia longa, A. recurvilabra, A. sinarum*)	Ma Dou Ling (fruit); Tian Xian Teng (herb), Qing Mu Xiang (root), Sei-Mokkou (Japanese), Birthwort, Long birthwort, Slender Dutchman's pipe
Aristolochia fangchi	Guang Fang ji (root), Fang ji, Fang chi, Mokuboi (Japanese), Kou-boui (Japanese), Kwangbanggi (Korean)
Aristolochia heterophylla	Han Fang Ji
Aristolochia indica	Indian birthwort (root), Yin Du Ma Dou Ling
Aristolochia kaempferi (Syn. *Aristolochia chrysops, A. feddei., A. heterophylla, A. mollis, A. setchuenensis, A. shimadai, A. thibetica, Isotrema chrysops, I. heterophylla, I. lasiops*)	Yellowmouth Dutchman's pipe, Zhu Sha Lian
Aristolochia macrophylla (Syn. *Aristolochia sipho*)	Dutchman's-pipe
Aristolochia manschuriensis (Syn. *Hocquartia manshuriensis*, Syn. *Isotrema manchuriensis*)	Manchurian birthwort, Manchurian Dutchman's pipe (stem) Guan Mutong (stem), Kan-Mokutsu (Japanese), Mokuboi (Japanese), Kwangbanggi (Korean)
Aristolochia maxima (Syn. *Howardia hoffmannii*)	Maxima Dutchman's pipe, Da Ma Dou Ling
Aristolochia mollissima	Wooly Dutchman's pipe, Mian Mao Ma Dou Ling
Aristolochia moupinensis	Moupin Dutchman's pipe, Huai Tong
Aristolochia serpentaria (Syn. *Aristolochia serpentaria*)	Virginia snakeroot, Serpentaria, Virginia serpentary
Aristolochia triangularis	Triangular Dutchman's pipe, San Jiao Ma Dou Ling
Aristolochia tuberosa	Tuberous Dutchman's pipe, Kuai Jing Ma Dou Ling
Aristolochia tubiflora	Tubeflower Dutchman's pipe, Guan Hua Ma Dou Ling
Aristolochia versicolar	Versicolorous Dutchman's pipe, Bian Se Ma Dou Ling
Asarum canadense (Syn. *Asarum acuminatum, A. ambiguum, A. canadense, A. furcatum, A. medium, A. parvifolium, A. reflexum, A. rubrocinctum*)	Wild ginger, Indian ginger, Canada snakeroot, False coltsfoot, Colic root, Heart snakeroot, Vermont snakeroot, Southern snakeroot, Jia Na Da Xi Xin
Asarum himalai(y)cum	Tanyou-saishin (Japanese)
Asarum splendens	Do-saishin (Japanese)

Other botanicals known or suspected to contain aristolochic acid are *Aristolochia argentina, A. baetica.* (Syn. *A. bracteolata*), *A. chilensis, A. cinnabarina, A. elegans (*Syn. *A. hassleriana), A. esperanzae, A. fimbriata, A. kwangsiensis* (Syn. *A. austroszechuanica), A. maurorum, A. rigida. A. rotunda, A. watsoni(i)* (Syn. *A. porphyrophylla), A. westlandi(i), A. zollingeriana* (Syn. *A. kankauensis, A. roxburghiana, A. tagala, Hocquartia kankauensis), Bragantia wallichii*.

Table 2 | Botanicals that may be adulterated with aristolochic acid and their respective vernacular names[55–59]

Botanical name	Common or other names
Akebia spp.	Akebia, Mu tong, Ku mu tong, Zi mutong, Bai mu tong, Mokutsu (Japanese), Mokt'ong (Korean)
Akebia quinata (Syn. *Rajania quinata*)	Chocolate vine, Fiveleaf akebia, Bai Mu Tong (stem), Mu Tong Gen (root), Yu zhi zi (seed), Mokutsu (Japanese)
Akebia trifoliata	Bai Mu Tong (stem), Bai Mu Tong Gen (root), Bai Mu Tong Zi (seed), Three leaf akebia, Yu zhi zi, San Ye Mu Tong Zi (seed), San Ye Mu Tong Gen (root)
Asarum caudatum	Wild ginger (root, leaves)
Asarum forbesii	Batei-saishin (Japanese)
Asarum heterotropoides (Syn. *Asarum heterotropoides*)	Keirin-saishin (Japanese) Chinese wild ginger, Manchurian wild ginger, Bei Xi Xin, Xin xin
Asarum sieboldii (Syn. *Asarum sieboldii*, *A. sieboldii* var. *seoulensis*, *A. heterotropoides* var. *seoulense*, *A. sieboldii*)	Usuba-saishin (Japanese) Chinese wild ginger, Xi Xin, Hua Xi Xin, Manchurian wild ginger, Siebold's wild ginger
Clematis spp.	Clematis, Mufangji, Clematidis, Ireisen (Japanese), Wojoksum (Korean)
Clematis armandii (Syn. *Clematis armandii* fo. *Farquhariana*, *C. armandii* var. *biondiana*, *C. biondiana*, *C. ornithopus*)	Armand's clematis Chuan Mu tong (stem), Xiao mu tong, Armand's virgin bower
Clematis chinensis	Chinese clematis, Wei Ling Xian
Clematis montana (Syn. *Clematis insulari-alpina*)	Chuan Mu Tong (stem)
Cocculus spp.	Cocculus
Cocculus indicus (Syn. *Anamirta paniculata*)	Indian cockle, Yin Du Mu Fang Ji
Cocculus orbiculatus (Syn. *Cissampelos pareira*)	Mu Fang Ji
Cocculus orbiculatus (Syn. *Cocculus cuneatus*, *C. sarmentosus*, *C. sarmentosus* var. *linearis*, *C. sarmentosus* var. *pauciflorus*, *C. sarmentosus* var. *stenophyllus*, *C. thunbergii*, *C. trilobus*, *Menispermum orbiculatus*, *M. trilobum*, *Nephroia sarmentosa*) Moku-boui (Japanese)	Mu Fang Ji (root)
Cocculus palmatus	Columba, Columbo
Cocculus palmatus (Syn. *Jateorhiza miersii*)	Colombo
Cocculus pendulus (Syn. *Cebatha pendula*, *Epibaterium pendulus*, *Cocculus epibaterium*)	Chui Mu Fang Ji
Cocculus trilobus	Mu Fang Ji (root)
Diploclisia chinensis	Xiangfangchi
Saussurea lappa	Mokkou (Japanese)
Sinomenium acutum (Syn. *Cocculus diversifolius* var. *cinereus*, *C. heterophyllus*, *Menispermum acutum*, *Sinomenium acutum* var. *cinereum*, S. *diversifolium*)	Orientvine, Xunfengteng, Dafengteng, Daqingmuxinag, Zhuigusan, Da ye qingshener, Mufangji, Hanfangji, Tuteng, Zhuigufeng, Maofangji
Stephania spp.	Stephania
Stephania tetrandra	Fen fang ji, Fang ji (root), Han fang ji (root), Kanboi (Japanese), Hanbanggi (Korean), Fun-boui (Japanese)
Vladimiria souliei	Sen-mokkou

Other botanicals that may be adulterated with aristolochic acid are: *Clematis hexapetala, Clematis uncinata*, (Syn. *Clematis alsomitrifolia, C. chinensis var. uncinata, C. drakeana, C. floribunda, C. gagnepainiana, C. leiocarpa, C. ovatifolia, C. uncinata var. biternata, C. uncinata var. coriacea, C. uncinata var. floribunda, C. uncinata var. ovatifolia, C. uncinata var. taitongensis*), *Cocculus carolinus* (Syn. *Cebatha carolina, Epibaterium carolinum, Menispermum carolinum*), *Cocculus diversifolius* (Syn. *Cocculus madagascariensis*), *Cocculus hirsutus* (Syn. *Cocculus villosus, Menispermum hirsutum*), *Cocculus laurifolius* (Syn. *Cinnamomum esquirolii*), *Cocculus leaebe, Cocculus madagascariensis* (Syn. *Cocculus diversifolius*), *Cocculus thunbergii, Diploclisia affinis* (Syn. *Diploclisia chinensis, Cocculus affinis*), *Menispernum dauricum*.

several in Asia, the risk of their mistaken use in traditional Chinese medicine is still high and could lead to a major concern for public health. Considering this fact, a reasonable way to decrease this risk should be the systematic quality control of herbal preparations by using reproducible and accurate analytical methods, such as high-performance liquid chromatography, liquid chromatography/mass spectrometry, or capillary electrophoresis.[61,62]

Interestingly, Mani[63] reported in a series of 2028 Indian patients with chronic kidney disease that chronic interstitial nephritis was a frequent cause (27.8%). We can speculate that some of them are AAN, as Indian folk medicine used more than 7500 plant species, including *Aristolochia bracteata*, *Aristolochia tagala*, and *Aristolochia indica*.[26] The identification of AA-related specific adducts on renal tissue could confirm this hypothesis.[32]

AA-ASSOCIATED UROTHELIAL MALIGNANCIES

Clinical findings

The striking association between AA exposure and the presence of urothelial abnormalities was described for the first time by Cosyns *et al.*,[29] who observed moderate atypia and atypical hyperplasia of the urothelium in four pieces of nephroureterectomies removed from three CHN/AAN patients before or at the time of transplantation. In the same time, two cases of urothelial carcinoma were reported among the Belgian cohort of CHN/AAN patients.[4,64] Considering the high risk for urothelial malignancy related to AA exposure, the prophylactic bilateral removal of the native kidneys and ureters was systematically proposed to CHN/AAN patients treated by dialysis or renal transplantation. It emerged that 40–45% of CHN/AAN patients displayed multifocal high-grade transitional cell carcinomas, mainly in

Table 3 | Products in which Mu Tong and Fang Ji are declared as ingredients[55,56]

Name
Ba Zheng Wan
Chun Yang Zheng Ji Wan
Da Huang Qing Wei Wan
Dang Gui Si Ni Wan
Dao Chi Wan
Dieda Wan
Fu Ke Fen Quing Wan
Guan Xin Su He Wan
Ji Sheng Ju He Wan
Kat Kit Wan
Long Dan Xie Gan Wan
Quell Fire
Shi Xiang Fan Shen Wan
Xin Yi Wan

the upper urinary tract.[33,65] The cumulative ingested dose of *Stephania* (in fact, *Aristolochia*) was demonstrated to be a significant risk for the development of urothelial carcinomas.[33]

A further follow-up of these CHN/AAN patients was performed in relation with the high risk of bladder carcinoma. Prospective screening cystoscopies were proposed to all renal-transplanted CHN patients from our Nephrology department. Among the 38 kidney recipients who accepted this follow-up (cystoscopy and bladder biopsies every 6 months), bladder urothelial carcinoma was diagnosed in 15 patients, 68–169 months after cessation of AA exposure (cumulative incidence 39.5%): eight urothelial carcinoma *in situ*, four noninvasive low-grade papillary urothelial carcinoma, and three infiltrating urothelial cancer. Out of 17 patients, 12 patients (71%) with a previous history of upper tract urothelial carcinoma developed bladder cancer during the follow-up, whereas this occurred only in three out of 21 (14%) patients free of upper tract urothelial carcinoma ($P<0.01$). Despite local and/or systemic chemotherapy, three patients died and two radical cystectomies had to be performed.[66]

Almost all of the cases of urothelial cancers were detected in CHN/AAN patients with end-stage renal disease. However, the case of a generalized urinary tract cancer without a significant renal failure after the intake of AA-containing Chinese herbal remedies showed that a dissociation between carcinogenicity and nephrotoxicity of AAs may occur.[67]

Other cases of urothelial carcinoma have been reported outside Belgium: in Taiwan, United Kingdom, France, and Hong Kong.[14,16,20,36,68,69] Interestingly, some of these cases, like those described by Laing, were reported after AA had been banned in the respective countries.

Taking into account all cases of AAN and urothelial malignancies reported worldwide, the Food and Drug Administration issued warnings to healthcare professionals, industry associations, and consumers regarding the safety of botanical products and dietary complements containing AA. The FDA recommended that all botanical remedies known or suspected to contain AA be discarded.[55,56] In 2002, the International Agency for Research on Cancer working group concluded that there was sufficient evidence in humans for the carcinogenicity of herbal remedies containing plant species of the genus *Aristolochia* (Group 1).[57]

AA activation, DNA-adducts formation, and carcinogenesis

Aristolochic acids I (AAI) and II (AAII), two structurally related nitrophenanthrene carboxylic acids, are the major components of the AA mixture contained in the plant extract of the *Aristolochia* species. Several enzymes have been demonstrated to metabolize AAI and AAII to a cyclic *N*-acylnitrenium ion with a delocalized positive charge able to covalently bind to the exocyclic amino groups of purine bases and to form DNA adducts.[40] The 7-(deoxyadenosine-N^6-yl) aristolactam I, 7-(deoxyguanosine-N^2-yl) aristolactam II and 7-(deoxy-adenosin-N^6-yl) aristolactam II are the main DNA adducts retrieved in AAN patients (Figure 2).[33]

Based on studies characterizing and quantifying the DNA adducts formed after modulation of metabolic pathways, the nitroreduction of AAs was demonstrated to be a crucial step leading to their ultimate DNA-binding species.[40] In human hepatic microsomes, the reductive activation of AA is mainly mediated by cytochrome P450 (CYP) 1A2, and to a minor extent by CYP1A1, whereas in human renal microsomes, NADPH/CYP reductase is more effective in AA biotransformation.[70,71] In addition, the NAD(P)H/quinine oxidoreductase and xanthine oxidase, two cytosolic enzymes found in human livers and kidneys, catalyze the activation of AAI to form DNA adducts.[72] Several factors such as drugs, smoking habit, environmental chemicals, and genetic polymorphisms affect the expression levels and activities of these enzymes, which could explain variations between individuals in the susceptibility to AA toxicity.

The phase II metabolism of AA consists of the presence in the urine and feces of AA metabolites in conjugated forms, such as glucuronides, sulfate, or acetate esters.[73,74] However, the precise role of conjugation enzymes in AA activation needs further investigation.[75]

The predominant 7-(deoxyadenosine-N^6-yl) aristolactam I *in vivo*, which is the most persistent DNA adduct detected in the target tissue, is a mutagenic lesion leading to AT→TA transversions. This specific mutation is retrieved at a high frequency in codon 61 of the H-*ras* protooncogene in tumors of rodents induced by AAI.[76] In AAN patients, an overexpression of P53 protein was observed in urothelial atypia and carcinomas, suggesting that the *p53* gene is also mutated.[65] Furthermore, DNA-binding studies demonstrated that AAI and AAII preferentially react with purine bases in the human *p53* gene, and the adduct distribution was not random.[77] A specific AAG-to-TAG mutation in codon 139 (Lys→Stop) of exon 5 in *p53* gene was detected in DNA isolated from one AAN-associated urothelial carcinoma.[78] These mutations in the *p53* gene probably trigger the tumorigenesis in AAN patients in the same way as the mutations in codon 61 of H-*ras* trigger the tumorigenesis in AA-intoxicated rodents. The metabolic activation of AA and its mediated carcinogenesis

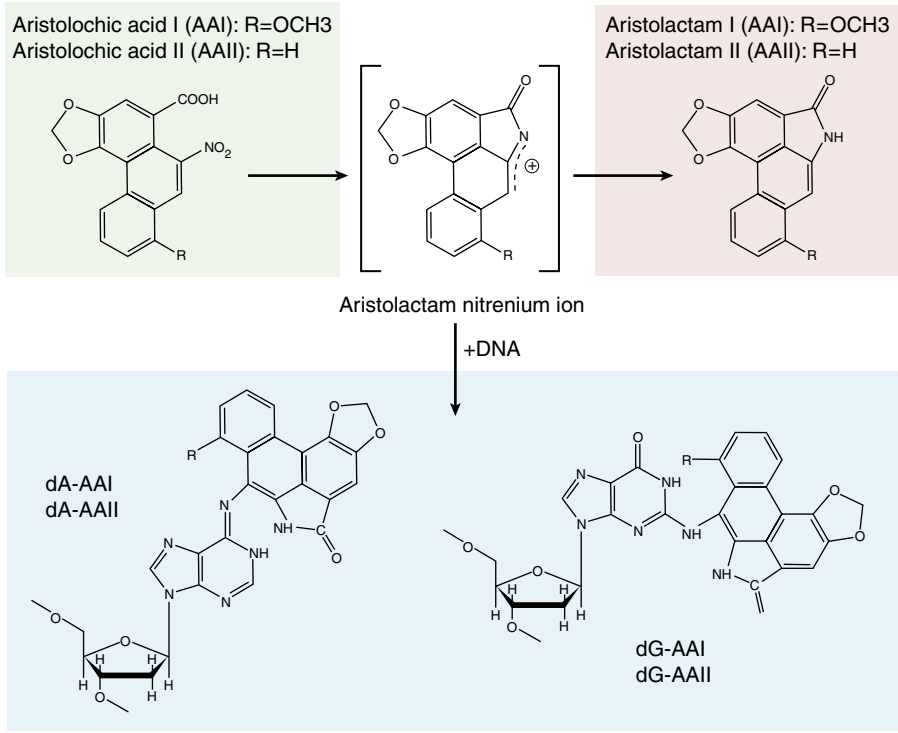

Figure 2 | Metabolic activation and DNA adduct formation of aristolochic acids I (AAI; R = OCH3) and II (AAII; R = H). The 7-(deoxyadenosine-N^6-yl) aristolactam I and II and the 7-(deoxyguanosine-N^2-yl) aristolactam I and II are formed after the reductive metabolic activation mediated by cytosolic reductases (NAD(P)H/quinone oxidoreductase, xanthine oxidase) as well as enzymes in hepatic (cytochrome P450 1A2 and 1A1, NADPH/CYP reductase) and renal microsomes (prostaglandin H synthase).

was the subject of an exhaustive and comprehensive review recently published by Stiborova *et al.*[75]

AA TOXICITY: EXPERIMENTAL STUDIES

Carcinogenic aspects

In the 1980s, Mengs and colleagues devoted several experimental studies to the toxicity of AA, especially to their carcinogenic properties. The administration by gavage of a mixture of AA (77% AAI and 21% AAII) to male and female Wistar rats at the dosage of 0.1, 1, and 10 mg per kg body weight per day for 3–12 months led to the development of forestomach carcinoma and urothelial dysplasia.[79] Following their initial observations, they examined the different steps of appearance and development of the AA-induced carcinoma in Wistar rats and NMRI mice.[80,81]

Just as CHN was frequently associated to urothelial dysplasia and malignancies, injections of AA to New Zealand white rabbits or male Wistar rats led to urothelial atypias.[34,35] In the rat model, papillary urothelial carcinoma and fibrohistiocytic sarcoma at the injection site were also retrieved.[35]

Nephrotoxicity aspects and animal models for AAN

The acute toxicity of AA was evaluated in Wistar rats and NMRI mice of both sexes (Table 4).[82] The lethal dose 50 (LD50) ranged from 56 to 203 mg kg^{-1} orally or 38 to 83 mg kg^{-1} intravenously, depending on species and sex. The histological evaluation revealed severe tubular necrosis, atrophy of the lymphatic organs, and large areas of superficial ulceration in the forestomach, followed by hyperplasia and hyperkeratosis of the squamous epithelium. According to the extensive tubular necrosis, the authors concluded that the animals died as a result of acute renal failure, although the renal functional parameters were not assessed.[82] In a subacute toxicity study, a daily oral administration of 25 mg AA per kg body weight to male Wistar rats induced after 4 weeks a moderate renal tubular necrosis with a significant glucosuria and proteinuria.[83] However, neither the proximal tubular atrophy nor the renal interstitial fibrosis, which are the typical histological findings for CHN, were reported in those studies.

Following the Belgian outbreak of CHN in 1993 and the etiopathological hypothesis of AA, new experimental studies were therefore undertaken. However, first attempts to experimentally reproduce CHN failed: two groups of seven Wistar rats were orally given either pure AAs (10 mg per kg for 5 days a week for 3 months) or AA-containing herbal powders mixed with fenfluramine. At the time of killing, animals in both groups developed the expected tumors but not renal tubulointerstitial fibrosis.[89] On the contrary, typical histological features of CHN/AAN, consisting of tubular atrophy, interstitial fibrosis, and urothelial atypias, were reproduced in 12 female New Zealand white rabbits after 17–21 months of intraperitoneal injections of 0.1 mg AA per kg body weight, 5 days a week.[34]

In the same time, we developed a short-term model for CHN by administrating subcutaneously 10 mg AA per kg body

Table 4 | Most relevant studies investigating the renal effects of aristolochic acid in animal models

Species	Dosage	Duration	AA components	Renal findings	Reference
Rat/mice	38–86 mg kg^{-1} IV or 150–300 mg kg^{-1} orally	Once	AAI (77%)/AAII (21%)	Severe PT cells necrosis. LD50 ranged from 56 to 203 mg kg^{-1} orally or from 38 to 83 mg kg^{-1} i.v., depending on species and sex.	82
Rat	0.2; 1.0; 5.0, or 25 mg kg^{-1} day^{-1} orally	28 days	AAI (77%)/AAII (21%)	Proteinuria and glucosuria. PT cells atypia and mild necrosis at highest dosage. Renal interstitial inflammatory cells infiltration.	83
Rat	10, 50, or 100 mg kg^{-1} orally	Once	AAI (77%)/AAII (21%)	At 100 mg kg^{-1}: ↑ sCr and BUN, proteinuria, ↑ γGT and NAG enzymuria. ↑ mitosis and necrosis of PT (*pars recta*)	84
NZW rabbit	0.1 mg kg^{-1} day^{-1}, 5 days a week	17–21 months	AAI (44%)/AAII (56%)	↑ sCr, glucosuria and low molecular weight proteinuria. Renal hypocellular interstitial fibrosis.	34
Rat	1 or 10 mg kg^{-1} day^{-1} s.c.	35 days	AAI (40%)/AAII (60%)	At 10 mg kg^{-1} day^{-1}: glucosuria, proteinuria, ↓ LAP enzymuria, and ↑ sCr on days 10 and 35. PT cells necrosis, mononuclear cells infiltrates (day 10), proximal tubular atrophy and interstitial fibrosis (day 35).	35
Mice	5 mg kg^{-1} day^{-1} i.p	14 days	AAI (44%)/AAII (56%)	During the regeneration phase (day 28), circulating transgene-derived HGF reduced AA-induced interstitial fibrosis, partially through a ↓ expression of TIMP-1 and ↑ MMP-9 activity.	85
Rat	10 mg kg^{-1} day^{-1} s.c.	35 days	AAI (40%)/AAII (60%)	RAS blockade reduced ED-1 + macrophage infiltration but not interstitial fibrosis induced by AA.	86
Mice	2.5 mg kg^{-1} day^{-1}, 5 days a week	14 days	AAI (55%)/AAII (45%) or AAI or AAII or Aristolactam I or AAIV	↑ sCr, glucosuria, proteinuria, proximal tubule injury, mononuclear cells infiltration, and interstitial fibrosis. Nephrotoxicity depending on strains (C3H/He > Balb/c > C57Bl6) and AA components (AAI > AAII). No nephrotoxicity of Aristolactam I and AAIV.	87
Rat	10 mg kg^{-1} day^{-1} s.c.	35 days	AAI (40%)/AAII (60%)	Defective activation of antioxidative enzymes and mitochondrial damage. Impaired regeneration and apoptosis of PT cells. Interstitial infiltration of monocytes/macrophages and CD8+ lymphocytes. Loss of epithelial markers concomitantly to *de novo* expression of mesenchymal cell markers and disruption of TBM.	88

Abbreviations: AA, aristolochic acid; BUN, blood urea nitrogen; γGT, gamma-glutamyltransferase; HGF, hepatocyte growth factor; i.p., intraperitoneal; i.v., intravenous; LAP, leucine aminopeptidase; LD50, lethal dose 50; MMP, matrix metalloproteinase; NAG, *N*-acetyl-β-D-glucosaminidase; NZW, New Zeeland white; PT, proximal tubule; RAS, renin–angiotensin system; s.c., subcuatenous; sCr, serum creatinine; TIMP, tissue inhibitor of metalloproteinase; TBM, tubular basement membrane.

wt per day to male Wistar rats.[35] On day 35, AA-treated rats displayed functional and histological renal impairment as a significant increase of serum creatinine and foci of severe proximal tubular atrophy surrounded by interstitial fibrosis. Nephrotoxicity of different components of AA was also studied in three strains of inbred male mice. The C3H/He mice intraperitoneally injected with 2.5 mg of AA per kg body weight, 5 days a week for 2 weeks, developed foci of proximal tubule cell injury surrounded by mononuclear cell infiltration on day 14.[87] Two weeks later, signs of proximal tubule cell proliferation were observed, whereas the inflammatory cell infiltration became more severe and interstitial fibrosis occurred.[87] In a CH3/He mice model, AAI exhibited a higher nephrotoxicity than AAII, which was also confirmed in *in vitro* studies on the proximal tubular LLC-PK1 cell line.[87,90,91]

Pathogenesis of AAN

The pathophysiological mechanisms by which AA induces renal interstitial fibrosis are still largely unknown. The treatment with an angiotensin-converting enzyme inhibitor ± angiotensin II receptor blocker did not modify the functional and structural renal impairments in AA-treated Wistar rats, suggesting that pathways leading to interstitial fibrosis seem to be independent of the renin–angiotensin system in this model.[86]

An early phase of acute tubular necrosis preceding the development of tubular atrophy and interstitial fibrosis was also observed in several experimental studies.[85,87,92] By using a transgenic mice model, Okada *et al.*[85] demonstrated that hepatocyte growth factor did not interfere with the acute phase but reduced the severity of interstitial fibrosis during the tubular regeneration phase, partially through a decreased expression of tissue inhibitor of metalloproteinase-1 and increased matrix metalloproteinase-9 activity.

Pozdzik *et al.*[88] recently showed in the rat AAN model that AA tubulotoxicity resulted in defective activation of antioxidative enzymes and mitochondrial damage. The progressive tubular atrophy was related to impaired regeneration of proximal tubular epithelial cells and apoptosis secondary to caspase-3 activation. The accumulation of vimentin and α-smooth muscle actin-positive cells in the interstitial areas expressing transforming growth factor-β suggested an increase in resident peritubular fibroblasts and their activation into myofibroblasts. These activated resident fibroblasts are proposed as the main source of collagen deposition during experimental AAN.[88]

As reported in several *in vivo* and *in vitro* studies, apoptosis is likely involved in the process of AA-induced proximal tubular atrophy.[85,90,93] In an *in vitro* study, LLC-PK1 cells exposed to AA displayed a rapid increase in their intracellular

calcium content leading to endoplasmic reticulum and mitochondrial stress, which in turn causes activation of the caspase pathway and finally apoptosis.[94] It should be noted that the potential impact of the AA–DNA adducts formation on the proximal tubular atrophy, for example, through a defect of DNA repair, is largely unknown and deserves further studies.

AAN AND BEN: THE TWO FACES OF JANUS?

Balkan-endemic nephropathy is characterized by chronic interstitial fibrosis with slow progression to end-stage renal disease and urothelial malignancy. It was first described about 50 years ago and affects residents of rural areas of Bulgaria, Bosnia, Croatia, Romania, and Serbia along the Danube river basin.[95]

As the etiology of BEN is currently unknown and different diagnostic criteria have been used in various countries, epidemiological data are difficult to compare. At least 25 000 individuals suffer from BEN or are suspected of having the disease, whereas the total number of people at risk in these countries may exceed 100 000. The significant epidemiologic features of BEN include (1) its focal occurrence in certain farming villages, with unaffected villages located in close vicinity; (2) a familial but not inherited pattern of disease, frequently affected members of the same household; (3) occurrence only in individuals who are older than 18 years, occurrence in $<10\%$ of households in endemic villages; and (4) a strong association with upper urinary tract urothelial carcinoma.[27]

A variety of environmental factors have been explored during the past 50 years, including heavy metals, arsenic, nitrogen species, silica, selenium deficiency, calcium and magnesium deficiency, polycyclic aromatic hydrocarbons in the water originating from Pliocene coal beds, viruses and bacteria, and mycotoxins.[96] Of these factors, ochratoxin A (OTA) has been the most investigated target of research.[97,98] Its presence in a variety of common foodstuffs, including cereal grains, was recognized more than three decades ago. OTA is classified by the International Agency for Research on Cancer[99] as a possible human carcinogen (Group 2B) on the basis on sufficient evidence for carcinogenicity in experimental animals but inadequate evidence in humans. OTA was shown to be a powerful rodent carcinogen, causing liver tumors in mice and renal tumors in mice (males) and rats, in particular adenomas and invasive carcinomas with elevated DNA ploidy distribution.[100,101]

Nephrotoxicity of OTA is also well recognized in animals. OTA induced the so-called porcine nephropathy after long-term exposure. Initially described in 1976 by Krogh, this renal disease is characterized by lesions compatible with those observed in chronic interstitial nephropathy, including proximal tubule injury and interstitial fibrosis.[95] However, as recently well summarized by Mally *et al.*[102], in all the remaining animal species studied (rodents), the most frequent histological observations are nuclear enlargement and polyploidy of proximal tubule cells, reflecting nuclear division without cytokinesis. No interstitial fibrosis has been reported associated or not with proteinuria, glucosuria, and increased creatininemia. Except in specific cases of acute tubular necrosis, no similarity could be found in the tubulo-interstitial compartment with histological lesions observed in AAN or BEN but, contrasting with BEN, interstitial fibrosis and tubular atrophy are not described.[102] Moreover, kidney tumors in OTA-exposed rats developed from the straight segment of the proximal tubule. No upper urinary tract carcinoma has been described, contrasting with its high prevalence in AAN and BEN.

Some studies found higher exposure (as measured by the intake of OTA and OTA levels in food stuff, blood, or urine) in individuals from endemic villages as compared with nonendemic villages (as reviewed by Stefanovic *et al.*; Long and Voice; Pfohl-Leszkowicz and Manderville; and Mally *et al.*).[27,98,102,103] OTA–DNA-related adducts (but also AA–DNA adducts) were detected in kidney tissue from patients with urothelial carcinoma or ureteral stenosis living in endemic areas and in approximately one-third of renal tissue samples from patients suffering from BEN and urothelial carcinoma.[27,40,98] The long-term persistence of such OTA–DNA adducts and even the methodological procedures used to detect them are still a matter of debate and may explain some discrepancy between data reported by different research groups.[97] Consequently, in the absence of a specific mutation profile related to OTA and direct DNA damage, some researchers like Turesky proposed an indirect mechanism involving oxidative stress and OTA-mediated cytotoxicity rather than direct genotoxic properties to explain OTA carcinogenicity.[104]

The so-called 'AA hypothesis' in BEN was initially formulated by Ivic in 1970.[105] He suggested a possible chronic dietary intoxication from bread made from wheat flour contaminated with seeds of *Aristolochia clematidis*. *Aristolochia* is, indeed, a common weed in wheat fields in endemic areas, and seeds are mixed with wheat grain during the annual harvest. This hypothesis seemed to be totally forgotten until the late 1990s.

In the first report of the Belgian CHN cases, the attention was already pointed out to the clinical similarities between this nephropathy and the Balkan endemic nephropathy.[1] In 1994, Cosyns *et al.*[29] underlined the hypocellular pattern of interstitial fibrosis decreasing from outer to inner cortex, which is a typical histopathological feature shared by both nephropathies. More recently, by using an ultrasensitive, quantitative ^{32}P-postlabeling method, in conjunction with HPLC and mass spectroscopic techniques, Grollman *et al.*[23] reported that dA-aristolactam and dG-aristolactam adducts were found in the DNA from the renal cortex of Croatian patients with BEN and in urothelial tumors from residents of BEN endemic villages. In addition, a predominance of A:T → T:A p53 mutations was detected in urothelial cancers from BEN patients (78%). This 'signature' mutation is rarely observed in transitional cell cancers (less than 5%).[77] Such mutation profile has never been found for OTA.

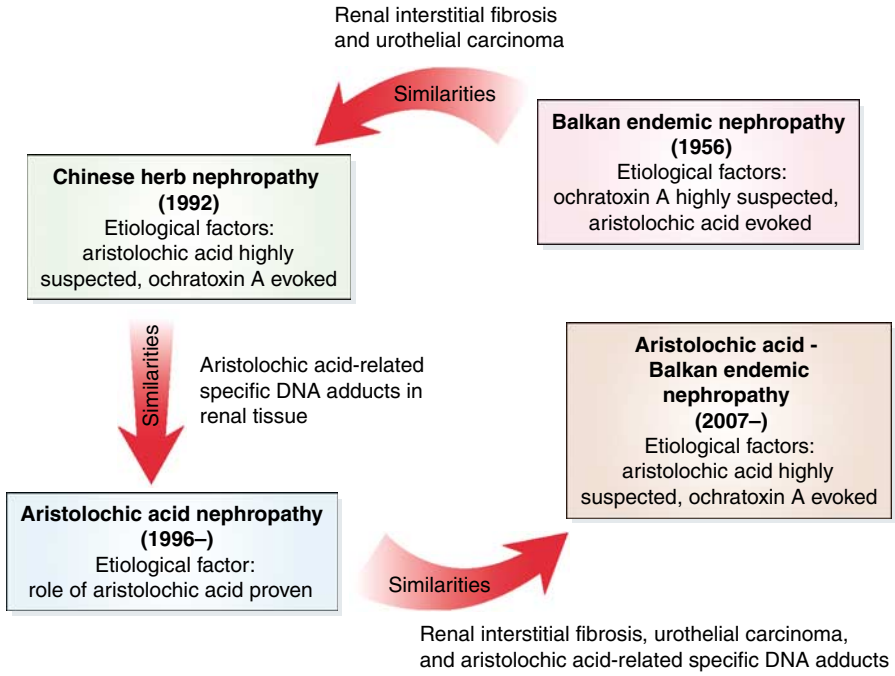

Figure 3 | Chinese herb/AAN and Balkan endemic nephropathy: the two faces of Janus?

Laboratory experiments performed by introducing the human p53 gene into mouse fibroblasts (Hupki cells) and treating them with AA resulted in the observation of a similar mutational A:T→T:A spectrum.[106,107] It was found to be similar to that reported in the H-*ras* gene of rodents treated with AA.[40]

Taken together, these clinical, histopathological, epidemiological, and recent toxicological data provide evidence that long-term exposure to AA may be the missing link to elucidate the complex multifactorial etiology of BEN (Figure 3).[108]

CONCLUSION

Often considered harmless, the regular use of herbal products may result in dramatic consequences as demonstrated by the 'Chinese-herb nephropathy' tragedy occurring in Belgium in the 1990s. The replacement of one substance (*Stephania*) by another more toxic compound (*Aristolochia*) was the cause of the outbreak of progressive renal fibrosis and urothelial carcinoma. Such substitution is probably responsible for a higher incidence of AAN than expected because this practice is common and allowed in traditional medicines that are based on a complex nomenclature using vernacular, not botanical, names. In addition, these products are still available legally in many countries and can be bought via the Internet. Finally, AA is proposed as the environmental causal factor for BEN affecting thousands of people living in the Danube basin.

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